Freeze NH (1995) Lectin affinity chromatography. *Protocols in Protein Chemistry*, pp. 901–919. New York: Wiley.
Gerard C (1990) Purification of glycoproteins. In: Deutscher MP (ed.) *Guide to Protein Purification, Methods in Enzymology*, vol. 182. New York: Academic Press.
Liu X-C and Scouten WH (1994) New ligands for boronate affinity chromatography. *Journal of Chromatography A*, 687: 61–69.
Liu X-C and Scouten WH (1996) Studies on oriented and reversible immobilization of glycoprotein using novel boronate affinity gel. *Journal of Molecular Recognition*, 9: 462–467.
West I and Goldring O (1996) Lectin affinity chromatography. In: Doonan S (ed.) *Methods in Molecular Biology, Protein Purification Protocols*, vol. 59, pp. 177–185. New Jersey: Humana Press.

Immobilized Metal Ion Chromatography

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Introduction

Since its introduction by Porath in 1975, immobilized metal ion affinity chromatography (IMAC) has developed into a robust and versatile tool. The number of uses is large and includes the isolation of metal-binding compounds from sea water, separation of enantiomeric forms of amino acids, tetracycline removal from animal products and protein purification. This article will focus on its application to protein purification, where it relies on the ability of certain amino acid side chains to form coordinative interactions with immobilized metal ion chelate complexes. As a chromatographic method it falls somewhere between biospecific affinity chromatography and ion exchange chromatography. The evolution of the technique, current tools and some specific technical details are discussed.

Background

Knowledge of the interaction of metal ions with proteins and the potential utility of immobilized metal chelators began during 1940–50, although it was not until 1974 that the method was first used to isolate a metalloprotein. The general use of IMAC was initiated in 1975 with a *Nature* publication from Porath. A summary of key milestones in the history of IMAC is presented in **Table 1**.

In the late 1970s and 1980s there were numerous publications on the choice of metals and investigations on the precise nature of the interactions that take place with proteins. It was assumed that surface-exposed residues were coordinating with the immobilized metal ions. Studies using free amino acids, peptides, and eventually engineered recombinant proteins, revealed the importance of certain amino acids – in particular histidine. Additionally, depending on the metal and chelating ligand employed, the spatial arrangement of the amino acids within the peptide or protein was also found to influence binding. This led to studies using model peptides with a wide range of histidine-containing sequences and in 1988 the first use of six consecutive histidine-residues as a purification tag (6His tag). In parallel with this 1987 saw the introduction of a metal–chelate complex with a high degree of selectivity for adjacent histidine residues (Ni^{2+}–NTA). Proteins purified using the 6His tag have been found to retain biological activity and their structures have also been solved by both X-ray and NMR – illustrating that, in the absence of metal ions, the tag has no defined secondary structure. Despite the enormous utility of the 6His tag the use of metal chelating ligand/metal combinations still has a role to play in the isolation of nontagged proteins from a wide variety of sources. The literature contains many examples of using IMAC as a one-step process to isolate native proteins, e.g. α-lactalbumin from milk and factor IX from blood. In addition, immobilized Fe^{3+} has been successfully used to separate phosphoproteins and immobilized Ca^{2+} to purify calcium-binding proteins.

The potential exists for even wider application to the separation of protein mixtures, with new chelators being introduced (e.g. TACN, see below).

Table 1 Key dates in the history of immobilized metal ion affinity chromatography

1974	First use of immobilized chelators to isolate metalloproteins
1975	First description of general technique (IMAC) using IDA
1983	Introduction of high performance on silica based media
1986	Use of Fe^{3+} chelates to purify phosphoproteins
1987	Introduction of NTA
1988	Introduction of genetically engineered His tags
1992	Introduction of TREN
1998	Introduction of TACN

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Table 2 Abbreviations, names and functional structures of chelators

Name	*Full name*	*Dentation*	*Structure*
IDA	Iminodiacetic acid	3	~ N(CH$_2$COOH)$_2$
TACN	1,4,7-Triazocyclonanane	3	~ N(CH$_2$CH$_2$NH$_2$CH$_2$CH$_2$
NTA	Nitrilotriacetic acid	4	~ CH(COOH)N(CH$_2$COOH)$_2$
TREN	Tris(2-aminoethyl)amine	4	~ NHCH$_2$CH$_2$N(CH$_2$CH$_2$NH$_2$)$_2$
Talon	Proprietary	4	Proprietary
TED	Tris(carboxymethly)ethylenediamine	5	~ N(CH$_2$COOH)CH$_2$CH$_2$N(CH$_2$COOH)$_2$

~ Indicates chosen form of linkage to a chromatographic support, usually with a suitable spacer.
– Indicates atoms involved in metal ion coordination.

Whether purifying proteins using native exposed histidines, post-translation modifications with phosphate or via an engineered 6His tag, IMAC provides a versatile and relatively gentle method with the potential to provide greater than 90% purity in a single chromatographic step.

Components

Metal Ions

A search of the literature on IMAC reveals a bewildering array of metal ions that have been used in this technique (e.g. Ag^+, Al^{3+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Eu^{3+}, Fe^{3+}, Hg^{2+}, La^{3+}, Mn^{2+}, Nd^{3+}, Ni^{2+}, Yb^{3+}, Zn^{3+}). The reason for this is that the nature of the metal ion (and indeed its chelator) influences the selectivity and affinity of the protein interaction. The most commonly used metals can be grouped into the 'hard' and 'soft' types – reflecting their electron orbital configuration and ability to act as electron acceptors. In free solution the metal ions exist with a shell of water molecules. Upon chelator or protein binding the water is displaced and a coordination bond to the metal ion is formed by the donation of free electron pairs from atoms in the chelator or in the amino acids (e.g. N, O and potentially S) of the protein. As such the atoms behave as monodentate ligands with the affinity estimated to be in the micromolar range. Both the protein and the immobilized chelator have the potential to be polydentate. For protein binding the 'soft' metal ions (e.g. Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}) show a preference for coordination with nitrogen-containing functional groups such as the imidazole of histidine (either δ or ε nitrogens). The 'hard' metal ions (e.g. Al^{3+}, Ca^{2+}, Fe^{3+}) show a preference for oxygen-containing groups such as carboxyls or phosphates found in phosphorylated proteins. These preferences are exploited in the nature and types of proteins purified with particular combinations of chelator and metal ion, and, to a certain extent, with the choice of buffer conditions. Within the 'soft' metal group a rank order of affinity for histidine residues has been established. In increasing strength of binding this order is $Co^{2+} \simeq Zn^{2+} < Ni^{2+} < Cu^{2+}$. Histidine is relatively rare, representing only 2.2% of the amino acids across all proteins with many containing none or none accessible on their surface. This provides a built-in selectivity for certain native proteins. The use of genetic engineering to introduce a 6His tag further exploits the selectivity for histidine. The preferred use of Ni^{2+} in IMAC with 6His tagged proteins is in part due to its higher coordination number ($Cu^{2+} = 4$, $Ni^{2+} = 6$) and the fact that the weaker binding potential of the Ni^{2+} is compensated for by the tag, thus providing an even greater degree of selectivity over other proteins from the recombinant host.

Chelating Ligands

Metal chelators bound to chromatographic media fix the metal ion to a solid support, enabling the separation process to take place. They modulate the affinity and selectivity of the chromatographic matrix as well as its capacity for proteins. A relatively large number of such ligands exists in the literature, although only a subset of these have found routine use in IMAC. This discussion limits itself to the most common chelating ligands and new developments in the area. **Table 2** presents a summary of the properties for a selection of chelating ligands.

During the design of chelating ligands several factors have been taken into consideration. Increasing the dentation number of the chelator will increase its affinity and reduce unwanted metal ion leakage from the column. Counterbalanced with this is the need to provide free coordination sites for the protein with binding capacity and affinity increasing as the number of these sites increases. In addition, metal ion transfer must be avoided, i.e. the chelating ligand

must bind the metal ion sufficiently tightly so as not to be stripped by proteins in the mixture to be purified.

As a consequence of the above considerations, several chelating ligands have been developed and successfully used in IMAC. **Figure 1** shows a schematic representation of the octahedral coordination of a metal ion (e.g. Ni^{2+}) with the chelators IDA, NTA and TED, illustrating the decrease in available protein-binding sites as the dentation of the chelator increases. IDA was the chelating ligand used by Porath in the first publication on IMAC in 1975. While adequate for the purpose, and still used today, this ligand is only tridentate and metal ion leakage can be a problem. When complexed with Cu^{2+} only one coordination site remains for protein binding. With Ni^{2+}, while three free coordination sites are available for protein binding, the metal binding is often too weak for practical use. For these reasons NTA was developed by Hochuli as an alternative to IDA. As shown in **Figure 2** the structure of NTA is closely related to that of IDA. NTA chelate with the oxygens of three carboxyl groups and a nitrogen, while IDA uses just two carboxyl groups and a nitrogen. The tetradentate nature of NTA means that metals other than Cu^{2+} must be used. When complexed with Ni^{2+}, two coordination sites are available for protein binding. The increased stability and coordination potential of NTA-based matrices has provided remarkable selectivity, especially when combined with recombinant proteins with engineered histidine tags. This combination was first introduced commercially by Qiagen.

To further address the issues of stability, selectivity and capacity, several alternative tetradentate

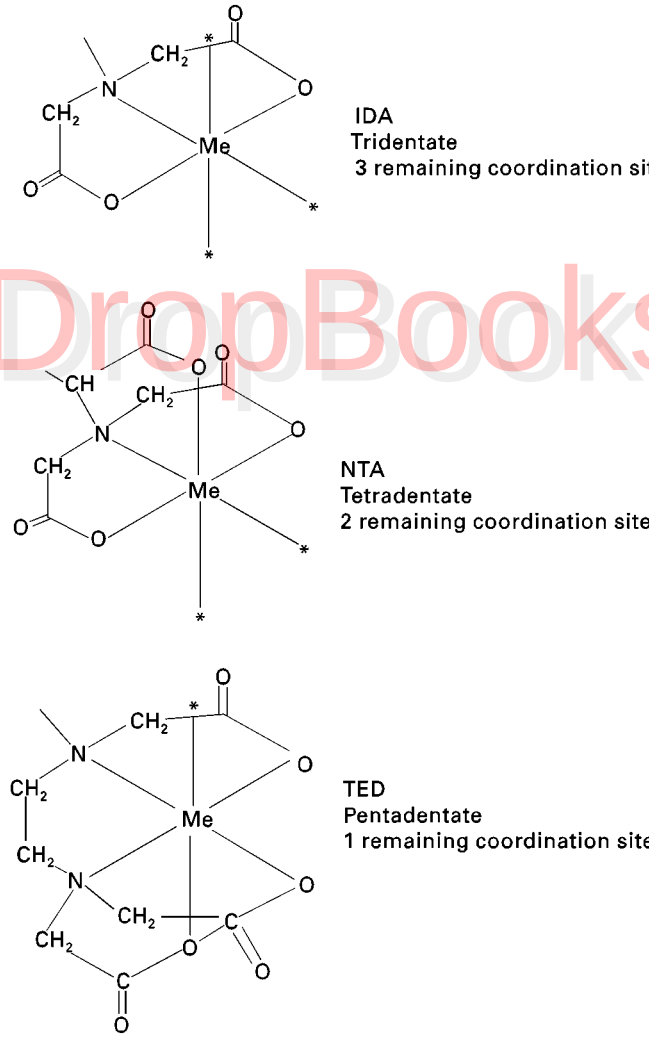

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Figure 1 Schematic representation of IDA and NTA metal chelation.

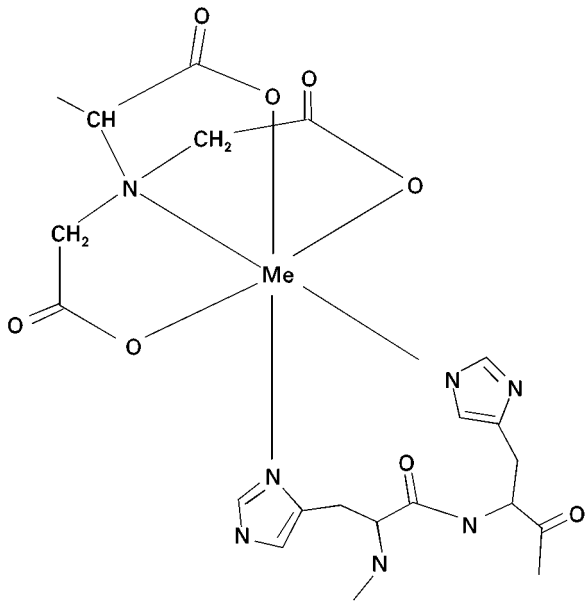

Figure 2 Ni^{2+}–NTA chelation and binding of consecutive histidine residues.

chelating ligands have also been developed and used successfully, e.g. TREN coupled to a high capacity matrix (Novarose-Inovata AB) for the rapid purification of goat immunoglobulins and Talon (Clontech) as a Co^{2+} loaded support which claims even higher selectivity in the isolation of 6His tagged proteins. TACN has recently been introduced and used with a range of 'soft' metal ions. This chelator exhibits remarkable metal-binding stability at low pH, where other chelators would exhibit loss of the metal. This extended pH range could be used to gain further selectivity. The pentadentate TED offers very tight metal ion binding and highly selective protein binding. In addition, the strength of metal ion binding to TED can be exploited as a second column to remove potentially leached metal ions from other IMAC eluates.

Media

The first commercially available IMAC medium was IDA-Sepharose (AP Biotech). Today, many IDA-chelating media are available, including modified forms of Sepharose (AP Biotech), agarose, polystyrene, polystyrene/divinylbenzene (Poros-Perseptive Biosystems), poly(alkalhydroxy-methacrylate), silica and even magnetic polystyrene beads (Dynabeads, Dynal Inc.). Among these types most are available as loose media and prepacked columns for either low pressure or high performance liquid chromatography. The commercially available NTA medium (Qiagen) and Talon (Clontech) are based on Sepharose CL-6B. At the time of writing, commercial media for TACN, TED and TREN are not available, although they can be made relatively easily using the chelator and commercially available activated media. In addition, membrane-based media can also be purchased (e.g. Sartobind IDA-Sartorius) or created via coupling of ligands to activated membranes.

Practical Considerations

The use of metal chelate chromatography should be relatively straightforward, i.e. charge with metal ions, wash, load with protein, wash and elute. However, as with most chromatographic processes there are a few points that may need close attention. In addition, the use of metal chelate chromatography has now gone beyond just the purification of proteins from crude mixtures to applications in protein folding, protein–protein interactions (footprinting) and immobilization of enzyme activities.

Protein Purification

Equipment Needs here can range from batch adsorption and elution, through very simple gravity-driven columns, to sophisticated pumping and control equipment for high performance methods. Since precise gradient mixing is not generally required, and many media present few problems with back pressure, IMAC is a relatively 'low-tech' process.

Buffers and loading conditions IMAC columns are compatible with a wide range of buffers, although those with the potential to act as chelators (e.g. citrate, Tricine) should be avoided. For the isolation of phosphoproteins on immbolized Fe^{3+} the use of phosphate buffers should be avoided. It should also be noted that phosphate buffers are not compatible with certain metals (e.g. Ca^{2+}) due to the formation of insoluble salts. The pH of the buffer will clearly be application dependent, although exposure of most columns to low pH (< 5) should be avoided since it will lead to loss of chelated metal ions due to protonation of the chelating groups. Any solutions containing imidazole should have the pH checked since imidazole can markedly alter the pH of 'buffered' solutions. Inclusion of a relatively high level of salt (e.g. 500 mM NaCl) is common practice in IMAC, serving to reduce nonspecific ionic interactions between the protein and the metal chelate complex. However, inclusion of such high levels of salt will also tend to increase nonspecific hydrophobic interactions with the column matrix. It is frequently best to ascertain the most suitable salt concentration on a case-by-case basis. The use of other chelating agents (e.g. EDTA, EGTA), often added as protease inhibitors, is also best avoided although separations

may be possible in the presence of ~1 mM concentrations if the load volume is relatively small compared to that of the column and/or the residence time is short. Reducing agents can also present a problem due to reduction of the metal ions, although low (10 mM) concentrations of β-mercapto ethanol may be tolerated by Ni^{2+}–NTA. Nonionic surfactants, low levels of organic solvents, 8 M urea and 6 M guanidine hydrochloride are generally compatible with IMAC. Temperature has little effect on the capacity of IMAC media.

When working with small volumes of load material or recombinant protein with a low accumulation level, the load volume/bed volume ratio becomes important. Deliberately overloading the column in such instances can improve the purity of the eluted material. In overloading the column the desired product, which binds with relatively higher affinity, will actively compete with weaker nonspecific interactions.

Certain components in common load materials from recombinant sources can introduce difficulties or potential contaminants. Insect cell media frequently contain high concentrations of histidine as a nutrient and can prevent the binding of 6His-tagged proteins being purified directly from the culture supernatant. Dialysis or dilution is required prior to loading. There is also a growing list of *Escherichia coli* proteins that regularly turn up as contaminants when purifying 6His-tagged proteins. These proteins include: chloramphenicol acetyl transferase from resistance selection: aspartate carbamoyl transferase; 30*S* ribosomal protein, rotamase and peptidyl prolyl *cis–trans* isomerase.

Washing Having loaded the media, in either batch or column mode, it is necessary to wash away unbound protein. This is generally achieved by washing with several bed volumes of loading buffer until the protein content of the wash material has reached an acceptable level. Low concentrations of eluting agents (e.g. imidazole) in the wash can help to improve the purity of the final product by eluting those components with relatively weak affinity. Inversely, the procedure of 'titration loading' can also be employed wherein weak binders are prevented from binding to the column by the inclusion of a low level of eluting agent in the loading buffer. Including a wash step with reduced salt can also serve to remove those potential contaminants bound to the medium via hydrophobic interactions. Proteins exhibiting such hydrophobic interaction include *E. coli*-derived proteases which, when concentrated along with target protein on the medium, can lead to severe degradation of the target. A low salt wash step can alleviate this problem.

Elution There are three potential ways of eluting protein from IMAC media:

1. Adding chelating agents that compete with the chelating ligand and the protein for metal ions.
2. Lowering the pH to protonate both the protein and the chelating groups on the chelating ligand, thus preventing metal ion chelation.
3. Introducing chelating agents that will compete with the protein for coordination to immobilized metal ion.

All three methods require further clean-up of the eluted material to remove unwanted components. Of the three methods, probably the least favourable is method 2 since this may lead to metal ion contamination of the eluted protein and retention of activity may not be compatible with low pH. Method 1 also strips the medium of metal ions, although in this instance they will be complexed with the added chelator. Method 3 is probably the most gentle form of elution. Imidazole mimics the coordination of histidine residues in the protein and can lead to effective elution when used in the tens to hundreds of millimolar range (**Figure 3** shows an example purification). For phosphoproteins immobilized on Fe^{3+} it is normal to use phosphate in the elution buffer and concentrations as low as 10 mM may be effective. The minimum concentration required should be determined on a case-by-case basis and cannot be simply predicted. Even for 6His-tagged proteins the minimum concentration of imidazole required for elution can vary by an order of magnitude depending on the target protein. In some instances a sharper elution profile can be obtained by inversion of the column prior to elution. Care should be taken regarding the potential effects of imidazole on the activity of the target protein (e.g. some protein kinases will appear to be inactive until the imidazole is removed). Additionally, protein precipitation can occur during removal of > 100 mM imidazole and upon thawing frozen samples.

Protein refolding The compatibility of IMAC with 8 M urea and 6 M guanidine hydrochloride has led to its use (primarily with 6His-tagged proteins) in refolding studies on immbolized protein. The potential advantage is that the protein is anchored to a solid support, thereby reducing aggregation that may be observed in even dilute solution refolding experiments. While no generic method is available this method has been successfully used to refold a growing number of proteins. In essence washing the immobilized protein on the IMAC column replaces conventional dialysis. With the protein immobilized in the presence of a strong denaturant, the level of

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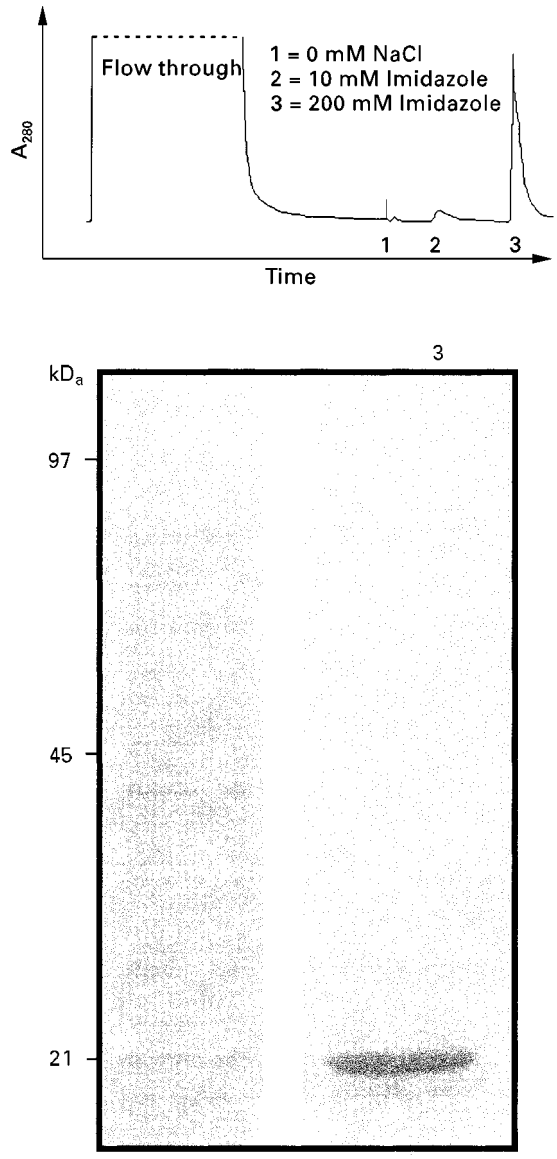

Figure 3 A 6His tagged fragment encoding residues 1–117 of human mdm2 (mdm2(1–177)-6His) was expressed in *E. coli* at ~1% total cell protein. Clarified lysate (L) was loaded on to a Ni^{2+}–NTA column in lysis buffer (50 mM Tris HCl, pH 8.0, 0.5 M NaCl, 10 mM β-mercapto ethanol, 1 mM PMSF, 1 $\mu g\ mL^{-1}$ leupeptin, 1 $\mu g\ mL^{-1}$ aprotinin, 1 $\mu g\ mL^{-1}$ pepstatin). After loading, the column was washed to baseline absorbance in the same buffer. Two washes were then performed: (1) 50 mM Tris HCl pH 7 and (2) as for (1) with 10 mM imidazole. Protein was then eluted by increasing the imidazole to 200 mM (3). The SDS-PAGE indicates that > 90% purity is obtained in this single chromatographic step.

denaturant is modulated in either a stepwise or gradient fashion. The refolded protein can then be eluted in a conveniently small volume.

Standard refolding protocols frequently employ dilute protein solutions and IMAC also provides a suitable method for concentrating the proteins during such processes. However, it should be noted that not all denaturants are compatible with IMAC (e.g. 400 mM arginine employed in arginine-assisted refolding interferes with binding of 6His-tagged proteins to Ni^{2+}–NTA).

Protein–protein interactions The binding of proteins to IMAC columns can also be used to study protein–protein interactions. Having bound one protein to the column, and washed away any excess, it is possible then to expose that protein to other proteins or mixtures to detect binding. This method can be used to 'footprint' a specific binding interaction or to detect binding partners in a complex mixture. If weak binders are of particular interest then it is also possible to minimize post-binding wash steps and directly run the SDS-treated beads on polyacrylamide gel electrophoresis.

Conclusion

The purification of proteins on immobilized metal ions is both effective and versatile. As well as a long standing role in the isolation of proteins with naturally available histidine residues, it has now become an everyday method for the isolation of 6His-tagged recombinant proteins. Recent reviews on the separation of phosphoproteins on immbolized Fe^{3+} indicate continued interest in such applications and no doubt additional uses will be found in the future.

See also: **I/Affinity Separation. II/Affinity Separation:** Theory and Development of Affinity Chromatography. **III/Enzymes:** Liquid Chromatography; **Proteins:** High-Speed Countercurrent Chromatography. **Appendix 1/ Essential Guides for Isolation/Purification of Enzymes and Proteins.**

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Further Reading

Anspach FB (1994) Silica based metal chelate affinity sorbents II. Adsorption and elution behaviour of proteins on iminodiacetic acid affinity sorbents prepared via different immobilisation techniques. *Journal of Chromatography* 676: 249–266.

Benson Chandra V (ed.) (1995) Current Protocols in Protein Science, Section 9.4. New York: Wiley.

Hermanson GT, Krishna Mallia A and Smith PK (eds) (1992) *Immobilised Affinity Ligand Techniques*, Section 3.1.5, pp 179–183. San Diego, USA: Academic Press.

Hochuli E, Dobeli H and Schacher A (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *Journal of Chromatography* 411: 177–184.

Hochuli E, Barnwarth W, Dobeli R, Gentz R and Stuber D (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate absorbent. *Bio/Technology* 6(11): 1321–1325.

Holmes LD and Schiller MR (1997) Immobilized iron(III) metal affinity chromatography for the separation of phosphorylated macromolecules: Ligands and applications. *Journal of Liquid Chromatography and Related Technologies* 20(1): 123–142.

Linder P, Guth B, Wulfing C, Krebber C, Steipe B, Muller F and Pluckthun A (1992) Purification of native proteins form the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols, *METHODS: A Companion to Methods in Enzymology* 4: 41–56.

Porath J (1992) Immobilised metal ion affinity chromatography. *Protein Expression and Purification* 3(4): 263–281.

Porath J, Carlsson J, Olsson I and Greta B (1975) Metal chelate affinity chromatography a new approach to protein-fractionation. *Nature* (*London*) 258(5536): 598–599.

Winzerling JJ, Berna P and Porath J (1992) How to use immobilised metal ion affinity chromatography. *METHODS: A Companion to Methods in Enzymology* 4: 4–13.

Wong JW, Albright RL and Wang N-HL (1991) Immobilized metal ion affinity chromatography (IMAC): chemistry and bioseparation applications. *Separation and Purification Methods* 20(1): 49–106.

Yip T-T and Hutchens TW (1994) Immobilized metal ion affinity chromatography. *Molecular Biotechnology* 1: 151–164.

Immunoaffinity Chromatography

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Introduction

Immunoaffinity chromatography is a general term that covers a range of techniques the use of which is now widespread. Often these are based upon the use of antibodies to a specific target molecule or macromolecule immobilized on some form of support (**Figure 1**). This is then used to separate or isolate the target molecule (or molecules of a similar structure) from a matrix in order to purify it for some subsequent purpose. Alternatively, immunoaffinity chromatography can be used to isolate antibodies by immobilizing the antigen, and indeed the first example of the use of the technique can be traced back to the pioneering work of Campbell *et al*. who, in 1951, immobilized bovine serum albumin to a derivatized cellulose in order to purify antibodies that had been raised to it (**Figure 2**). These immunologically-based methods include in addition immunoaffinity precipitation, immunoaffinity adsorption and immunoaffinity extraction. Indeed the use of the term 'chromatography' is perhaps something of a misnomer as the technique often corresponds more to the online extraction of the target molecule onto the sorbent. Following extraction, a wash step is used to remove unwanted material followed by the recovery of the desired molecule with a strong eluent. It could thus be argued that in many applications immunoaffinity chromatography is simply immunoaffinity extraction in a column format. The term 'immunoaffinity chromatography' is however, widely used and understood by its practitioners.

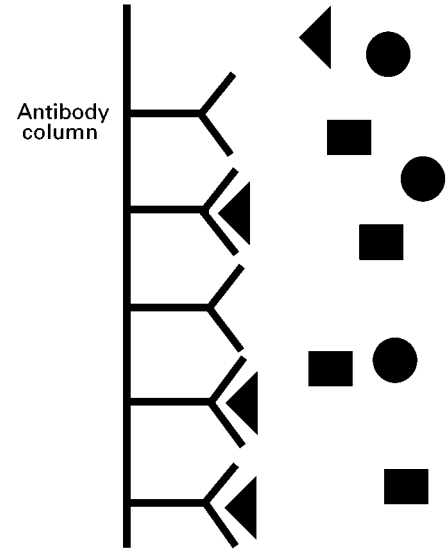

Figure 1 Stationary phase with antibodies bound, only the antigen to which the antibodies were raised is retained. Other molecules pass through with little or no retention.